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GENETIC STRUCTURING OF THE TEMPERATE GORGONIAN CORAL *CORALLIUM RUBRUM* ACROSS THE WESTERN MEDITERRANEAN SEA REVEALED BY MICROSATELLITES AND NUCLEAR SEQUENCES

Federica Costantini, Cécile Fauvelot, M. Abbiati

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1 Title: GENETIC STRUCTURING OF THE TEMPERATE GORGONIAN CORAL *CORALLIUM*
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4

5 Authors: Costantini F, Fauvelot C, Abbiati M
6

7 Address: Centro Interdipartimentale di Ricerca per le Scienze Ambientali and Dipartimento di
8 Biologia Evoluzionistica Sperimentale, University of Bologna, Via S. Alberto 163, I – 48100
9 Ravenna, Italy
10

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13

14 Corresponding author:

15 Federica Costantini

16 Via S. Alberto 163,

17 I – 48100 Ravenna, Italy

18 Tel. +39 0544 937401

19 Fax. +39 0544 937411

20 E-mail: federica.costantini@unibo.it
21

22 Running title: Genetic structuring of *Corallium rubrum*
23

24 **Abstract**

25

26 In the past decades, anthropogenic disturbance has increased in marine coastal habitats leading to
27 dramatic shifts in population size structure of various marine species. In the temperate region, the
28 gorgonian coral *Corallium rubrum* is one of the major disturbed species, mostly owing to the
29 exploitation of its red skeleton for jewellery purposes. Red coral is a gonocoric species inhabiting
30 subtidal rocky habitats in the Mediterranean and neighbouring Atlantic coasts. In order to
31 investigate the spatial genetic structuring of *C. rubrum*, five microsatellite markers and the
32 ribosomal internal transcribed spacer 1 (ITS-1) sequences were analysed in eleven samples from the
33 North Western Mediterranean Sea. Phylogenetic reconstructions obtained from ITS-1 sequences
34 analysis showed that samples from Minorca and Sardinia islands were the most divergent while the
35 three samples from the Tuscan archipelago (Argentario, Giannutri and Elba) appeared genetically
36 homogeneous. Both markers revealed a strong structuring over large spatial scales (though to a
37 different extent) with no isolation by distance pattern. Microsatellites based F_{ST} estimates appeared
38 much smaller than ITS-based estimates and not significantly correlated, likely due to microsatellite
39 allele size homoplasmy typical of these highly polymorphic loci. Our study shows that the absence of
40 clear patterns of genetic structuring over large spatial scales together with strong genetic structuring
41 should be interpreted with caution because such patterns may hide underlying small scale genetic
42 structuring. Our results further confirm that effective larval dispersal in red coral is highly restricted
43 in the North Western Mediterranean Sea, suggesting that an increase of anthropogenic disturbance
44 could aggravate the disappearance of red coral, not only along the Mediterranean coasts but also,
45 and with more intensity, in the main Mediterranean islands.

46

47 **Introduction**

48

49 In the past decades, human-induced disturbance (e.g. habitat loss and fragmentation, global climate
50 change, overexploitation and other effects due to fishing, pollution and tourism) has increased in
51 marine coastal habitats (Airoldi & Back 2007). Owing primarily to this disturbance, biogenic reefs
52 in tropical and temperate habitats have suffered long-term degradation affecting both species and
53 genetic diversities (Hellberg *et al.* 2002, Hughes *et al.* 2002, Pandolfi *et al.* 2003, Bellwood *et al.*
54 2004, Worm *et al.* 2006). Sustainable exploitation and conservation of marine species are among
55 the priorities of modern bio-resource management (Gray 1997). In this context, estimating
56 connectivity among populations is essential because dispersal is a key element warranting
57 population resilience following disturbance (Palumbi 2003, Bellwood *et al.* 2004).

58

59 *Corallium rubrum* is a gorgonian coral (Anthozoa, Gorgonacea) inhabiting subtidal rocky habitats
60 at a depth ranging from about 10 to 600 m (Taviani M, personal communication) in the
61 Mediterranean and neighbouring Atlantic coasts (Zibrowius *et al.* 1984). Due to the high economic
62 value of its red axial carbonate skeleton, red coral populations have been intensively harvested
63 (Santangelo & Abbiati 2001). This major source of disturbance may drive dramatic shifts in
64 population size structure and, eventually, lead to the extinction of local commercial banks
65 (Santangelo & Abbiati 2001, Garrabou *et al.* 2001). Furthermore, red coral populations are
66 vulnerable to water temperature rise (Cerrano *et al.* 2000; Garrabou *et al.* 2001). Therefore, red
67 coral is currently listed among the species of community interest of the European Union Habitat
68 Directive (92/43/EEC, Appendix V).

69

70 Within the Mediterranean Sea, the distribution of red coral is mainly restricted to the rocky shores
71 of the western Mediterranean basin, though Chintiroglou *et al.* (1989) reported records along the
72 Greek coasts of the North Aegean Sea. The distribution of the species shows major gaps related to
73 the presence of stretches of sandy shores (e.g. Gulf of Valencia, Gulf of Lion, Versilia plain, Gulf of
74 Gaeta). Moreover, red coral has a strong requirement towards its habitat, defined by the
75 morphology and biogenic nature of the substratum, as well as the range of abiotic variables (e.g.
76 light intensity, water temperature and turbidity, sediment loads, current regime) (Weinberg 1979).
77 As a consequence, the distribution of *Corallium rubrum* is highly fragmented at various spatial
78 scales.

79

80 Red coral is a gonocoric species, brooding lecithotrophic planulae (Weinberg 1979). In order to
81 investigate the effective larval dispersal of red coral in the western Mediterranean Sea, early
82 population genetics studies have been conducted using allozymes. They have shown the occurrence
83 of significant genetic divergences among samples at a distance of tens of kilometres (Abbiati *et al.*
84 1993). At shorter distances (about 200 m), no significant genetic structuring was observed using
85 allozymes (Abbiati *et al.* 1997). However, allozyme markers, due to their low mutation rate and
86 polymorphism may have a limited power in revealing a genetic structure at small spatial scales.
87 Indeed, recently, Costantini *et al.* (2007) using four microsatellite loci specifically developed for *C.*
88 *rubrum* (Costantini & Abbiati 2006) have found a strong genetic structuring at spatial scales of tens
89 of meters among samples within two locations on the Ligurian Sea. These patterns are explained by
90 the reduced swimming ability and geonegative behaviour of the planulae, together with a short
91 larval duration (estimated from 4 to 12 days under laboratory conditions, Vighi 1972, Weinberg
92 1979), which suggest that, once released, larvae settle in close vicinity of the parental colonies
93 (Vighi 1972). Additionally, differences in genetic variability and in F_{ST} estimates between locations
94 (Costantini *et al.* 2007) suggested that environmental features (e.g. habitat characteristics,

95 geomorphology, hydrodynamics) strongly influence the genetic structure of red coral populations at
96 larger geographical scales.

97
98 In order to further investigate the spatial genetic structuring of *C. rubrum*, eleven populations within
99 the distribution range of the species in the North Western Mediterranean Sea have been analysed.
100 Since mitochondrial DNA did not reveal intraspecific variation in red coral (Costantini *et al.* 2003),
101 likely due to its slow evolutionary rate as observed in anthozoans (Romano & Palumbi 1997,
102 Shearer *et al.* 2002, Hellberg 2006), in this study, two nuclear molecular markers with differing
103 levels of polymorphism have been used. Allele frequencies at five microsatellite loci have been
104 analysed in *C. rubrum* samples. Since microsatellites previously revealed genetic structuring at very
105 small scales (Costantini *et al.* 2007), sequences of the internal transcribed spacer 1 region (ITS-1) of
106 the nuclear ribosomal DNA were also analysed in order to test for homoplasy at microsatellite loci
107 over large scales. The results of this study may help understanding how small scale processes may
108 influence patterns over larger spatial scales in *Corallium rubrum* populations, as well as being
109 useful to conservation and sustainable management strategies of the species at the Mediterranean
110 scale.

111 112 **Materials and methods**

113 114 *Sample collections and molecular analysis*

115 Red coral colonies were collected by SCUBA diving at 11 locations along the Mediterranean
116 coasts, between 25 and 30 m depth (Figure 1). Shoreline distance among samples ranged from 30
117 km (between Argentario and Giannutri) to about 2650 km (between Medes and Korcula). At each
118 location, branch fragments from 20 to 50 individual colonies were collected and preserved in 80%
119 ethanol at 4°C.

120
121 Total genomic DNA was extracted from 2-4 polyps per individual colonies using a cetyltrimethyl
122 ammonium bromide (CTAB) protocol (Winnepennickx *et al.* 1993) with standard phenol-
123 chloroform-isoamyl alcohol (25:24:1) extraction. Five microsatellite loci (COR9, COR15, COR48
124 and COR58, used in Costantini *et al.* (2007), and COR46) specifically developed for *Corallium*
125 *rubrum* were amplified (Costantini & Abbiati 2006). Genotyping was carried out on an ABI 310
126 Genetic Analyser, using forward primers labelled with 6-FAM, HEX or TAMRA (MWG Biotech)
127 and ROX HD400 (Applied Biosystems) as internal size-standards. Allele sizing was performed
128 using GeneScan Analysis Software version 2.02 (Applied Biosystems).

129
130 Polymerase chain reaction (PCR) amplifications of the ITS-1 region were carried out using the
131 primers ITS1-new-F 5'-TCGTAACAAGGTTTCCGTA-3' and ITS1-new-R 5'-
132 TAACGGTGGATCTCTTGGCT-3' (Costantini 2001). Each 25 µl PCR reaction contained
133 approximately 20 ng DNA, 1X PCR buffer (Promega), 2 mM MgCl₂, 0.5 µM of each primer, 0.4
134 mM dNTPs and 1 U of *Taq* polymerase (Promega). Amplifications were performed on a GeneAMP
135 PCR System 2700 (Applied Biosystems) as follows: an initial denaturation at 95°C for 3 min, 30
136 cycles including 95°C for 30s, 57°C for 30s and 72°C for 60s. A final extension at 72°C for 7 min
137 was added. Amplified fragments were purified with an ExoSAP-IT kit (Amersham Pharmacia) and
138 cycle sequenced in both directions using an ABI Prism Big Dye Terminator Cycle Sequencing kit
139 (Applied Biosystems). Sequences were run on an ABI 310 Genetic Analyser (Applied Biosystems).
140 Given that the cloning of PCR products is costly and laborious, and, additionally, artefacts can be
141 produced during the cloning step due to *in vitro* recombination upon transformation of bacterial
142 cells with heteroduplex DNA (Zhang & Hewitt 2003; Vollmer & Palumbi 2004), all ITS-1

143 fragments were directly sequenced without prior cloning. In order to avoid the term “haplotype”,
144 which represents an haploid component of a given sequence, the term “sequence type” was used to
145 refer to every distinct type of ITS-1 sequence detected, as proposed by Worheide *et al.* (2002).
146

147 *Genetic variability*

148 Microsatellite diversity within samples was estimated using El Mousadik & Petit (1996) allelic
149 richness (A) in FSTAT version 2.9.3.2 (Goudet 2001), and observed (H_{obs}) and Nei 's (1987)
150 unbiased expected heterozygosity (H_{exp}) in GENETIX software package version 4.03 (Belkhir *et al.*
151 2004). All loci were tested for linkage disequilibrium using GENEPOP version 3.4 (Raymond &
152 Rousset 1995) as implemented for online use (<http://genepop.curtin.edu.au/>). Single and multilocus
153 F_{IS} were estimated using Weir & Cockerham's (1984) fixation index and, because most of F_{IS} were
154 positive ($H_{obs} < H_{exp}$), deviations from Hardy-Weinberg equilibrium (HWE) were tested using
155 Fisher's exact test, using the null hypothesis $H_0 =$ no heterozygote deficiency, with the level of
156 significance determined by a Markov-chain randomization (1000 dememorizations, 100 batches,
157 and 1000 iterations per batch) in GENEPOP. Significance levels for multiple comparisons of loci
158 across samples were adjusted using a standard Bonferroni correction (Rice 1989). The presence of
159 null alleles was examined by estimating null allele frequencies for each locus and sample following
160 the Expectation Maximization (EM) algorithm of Dempster *et al.* (1977) using FREENA (Chapuis &
161 Estoup 2007).
162

163 ITS-1 sequences were edited and aligned manually using BIOEDIT version 7.0.4 (Hall 1999).

164 Genetic diversity within samples was estimated using sequence type diversity (h , Nei 1987) and
165 nucleotidic diversity (π , Nei 1987) using ARLEQUIN ver 3.1 (Excoffier *et al.* 2005).

166 Since ITS regions have frequent insertions/deletions (indels) that may be phylogenetically
167 informative (Vogler & DeSalle 1994) and may originate from single evolutionary events regardless
168 of their size (Girabet & Wheeler 1999), alignment gaps were treated using a conservative approach.
169 Here, each indel with a different start and/or end position (i.e. not point mutations), which all
170 sequence type either have or lack, was recoded as a new single base and added at the end of the
171 sequence (see Table 3), and gaps were further treated as missing nucleotides.
172

173 Hierarchical likelihood ratio tests were used to determine the best-fit model of nucleotide
174 substitution using the program MRMODELTEST version 2.2 (Nylander 2004). The Jukes & Cantor
175 (JC) model (equal nucleotide frequencies and equal rate of nucleotide substitution between
176 nucleotides) was supported by the likelihood ratio test ($P < 0.000001$) and was then used in
177 following analysis. Phylogenetic trees of the relationships among sequence types were constructed
178 using Maximum parsimony (MP) in PAUP 4.0b10 (Swofford 2002) and Bayesian inference (BI)
179 using MRBAYES 3.1 (Ronquist & Huelsenbeck 2003). Maximum parsimony analysis was performed
180 under the heuristic search option employing random stepwise addition, 10 random additions and
181 TBR branch swapping. Nodal support was measured using a non parametric bootstrap method with
182 100 pseudo replications. BI analysis was performed using one cold chain and three incrementally
183 heated chains with $T = 0.1$. Starting trees for each chain were set as random and all settings were
184 default values of MRBAYES unless stated. Each Metropolis-coupled Markov chain Monte Carlo
185 (MCMCMC) was run for 5 million generations, with trees sampled every 1000 generations, and the
186 first million generations were discarded. Results were summarized as 50% majority rule consensus
187 trees including compatible groupings and branch lengths. Posteriors probabilities were used to asses
188 clade support. Trees were displayed using the software TREEVIEW version 1.6.0 (Page 1996).
189

190 *Clustering analysis*

191 The number of clusters detected among samples, K , was estimated by employing a Bayesian
192 approach implemented in the program STRUCTURE version 2.2 (Pritchard *et al.* 2000, Falush *et al.*
193 2003, 2007). Each individual was assigned to probable common clusters based on the similarity of
194 their multilocus genotypes at five microsatellite loci. Mean and variance of log likelihoods of the
195 number of clusters for $K=1$ to $K=12$ were inferred from multilocus genotypes by running
196 STRUCTURE 5 times with 500 000 repetitions each (burn in = 50 000 iterations) under the admixture
197 ancestry model and the assumption of correlated allele frequencies among samples as suggested in
198 Falush *et al.* (2003). The mean membership of each individual described the likelihood of that
199 individuals belonging to the respective clusters. Following the recommendations of Evanno *et al.*
200 (2005), we calculated the ad hoc statistic ΔK based on the rate of change in the log likelihood of
201 data between consecutive K -values. Due to the presence of null alleles, the clustering analysis was
202 conducted on the original dataset (not in HWE), using the option of null alleles coded as recessive
203 alleles described in Falush *et al.* (2007). Indeed, we could not use a dataset adjusted for the presence
204 of null alleles such as the so-called INA method described in Chapuis & Estoup (2007) since this
205 procedure consider only one null allele common to all populations, which may biased the
206 assignment tests (Chapuis & Estoup 2007, Chapuis personal communication).
207

208 *Population structure analysis*

209 Genetic divergence among samples at microsatellite loci was estimated in FREENA using the F_{ST}
210 estimates of Weir (1996) and following the so-called ENA method described in (Chapuis & Estoup
211 2007) which provides unbiased F_{ST} estimates, computed excluding null alleles. Pairwise F_{ST} among
212 samples were also estimated using the original dataset (not corrected for nulls). Genotypic
213 differentiation among samples was tested with an exact test (Markov chain parameters: 1000
214 dememorizations, followed by 1000 batches of 1000 iterations per batch) with the original dataset,
215 and the P -value of the log-likelihood (G) based on the exact test (Goudet *et al.* 1996) was estimated
216 in GENEPOP. For the ITS-1 sequences dataset, genetic differentiation for each pairwise sample was
217 estimated using Φ statistics (Φ_{ST} based on haplotype frequencies and molecular divergence, using
218 JC model) and its significance determined using a permutation test (10 000 permutations) in
219 ARLEQUIN. Sequential Bonferroni corrections (Rice 1989) for multiple comparisons were applied.
220

221 For both microsatellite and ITS-1 sequences datasets, isolation by distance model between samples
222 was tested through a Mantel test (Mantel 1967) computed using the ISOLDE program implemented
223 in GENEPOP. A significant correlation between genetic differentiation estimates and the logarithm of
224 the geographic distances among samples was tested using 1000 permutations within the original
225 matrices.
226

227 An analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) implemented in ARLEQUIN was
228 conducted to examine the partition of the genetic variance among red coral samples. With this
229 purpose, red coral samples were grouped A) according to their sea of origin (six groups: Balearic
230 Sea, Sardinian Sea, Ligurian Sea, North and Southern Tyrrhenian Sea, Adriatic Sea), B) based on
231 the existing gaps in the distribution of the species (seven groups: Minorca; Sardinia; Medes; Plane
232 and San Fruttuoso; Calafuria, Argentario, Giannutri and Elba; Palinuro; Korcula), C) *a priori*, based
233 on the ITS-1 phylogenetic tree (four groups: Minorca; Sardinia; Tuscan archipelago samples and all
234 the other samples, see results), and D) *a priori*, based on the results of the clustering analysis (two
235 groups: Medes, Minorca, Plane, Sardinia, San Fruttuoso, Calafuria and Korcula; Argentario,
236 Giannutri, Elba and Palinuro).
237

238 **Results**

239 *Microsatellite loci variability and null alleles*

240 Over all samples, locus COR15 exhibited the lowest variation (9 alleles in total), while COR9 had
241 the largest number of alleles (33). Overall, observed heterozygosities ranged from 0.26 (in COR9
242 and COR46) to 0.45 (in COR15 and COR48) and expected heterozygosities ranged from 0.66 (in
243 COR15) to 0.93 (in COR9). No linkage disequilibrium was detected among loci (all $P > 0.05$ after
244 Bonferroni corrections) and all loci were therefore considered genetically independent. *Corallium*
245 *rubrum* samples showed considerable variation in their allelic richness over all five microsatellite
246 loci, ranging from 5.1 for Giannutri to 10.0 for Minorca (Table 1). Significant multilocus deviations
247 from Hardy-Weinberg expectations (HWE) were observed in all samples (Table 1). Multilocus
248 estimates of F_{IS} ranged from 0.15 to 0.71, showing in all cases heterozygote deficiencies.
249 Significant deviations from HWE were also observed in nearly all samples for each locus, with the
250 exception of COR15, the less polymorphic one. Assuming HWE, estimated null allele frequencies
251 (R) ranged among loci from 0 to 0.78 (Table 1). Within each locus, the number of expected null
252 homozygotes within samples based on HWE ($N \cdot R^2$) was significantly higher than the average
253 number of observed null homozygotes for COR9, COR46 and COR48 (paired t-test, $P < 0.001$, $P =$
254 0.006 and $P = 0.012$ respectively). Over all samples, the average number of expected null
255 homozygotes per locus was significantly higher than the average number of observed null
256 homozygotes (paired t-test, $P = 0.035$).

257

258 *ITS-1 sequence variation and phylogenetic analysis*

259 Intra-individual polymorphism was detected in 17.5% of the 280 amplified individuals (through the
260 presence of 1-3 doublet chromatogram peaks or partially unreadable sequences relatives to indels).
261 This polymorphism was distributed unequally among samples, with a number of failed sequences
262 ranging from 0 in Plane and Argentario samples to 15 in Giannutri sample (Table 2). Individuals
263 showing such polymorphism in at least one nucleotide position were excluded for further analysis,
264 reducing the total number of analyzed individuals to 231.

265

266 Across all 231 individuals, the length of the amplified ITS-1 fragment varied among individuals
267 between 228 and 266 bp due to the presence of numerous indels (Table 3). Single base pair indels
268 were mostly associated with the presence of homopolymers but not with microsatellite repeat
269 regions as observed in ITS1 for *Acropora* (van Oppen *et al.* 2000, Vollmer & Palumbi 2004).
270 Including all gaps, the alignment of all individual sequences consisted of 278 positions of which 87
271 were variable, corresponding to 35 nucleotide substitutions and 52 indels. The sequence alignment
272 (Table 3) showed two highly variable blocks of indels and minor stutters. The first large indel, from
273 158 to 188 bp, correspond to a 31 bp deletion that was observed only in the Tuscany archipelago
274 samples (Argentario, Giannutri and Elba; sequence types 30, 31, 32, and 45, Table 2). The second
275 highly variable region consist of two smaller indels, situated between 210 to 222 bp and between
276 244 and 246 bp, that were found only in Minorca sample (sequence types from 10 to 15, Table 2).
277 Out of the 51 different sequence types revealed, nine were found in more than one sample
278 (sequence types 1-5-6-17-29-32-42-49-50, Table 2). Sequence type 5 was the most widespread
279 (seven samples out of 11), though not the most abundant one (12%; Table 2). The most abundant
280 sequence type (sequence type 17, 18.6%) was found only in Plane and Calafuria. The remaining
281 sequence types were unique to a single location and often found in a single individual (28 singletons
282 in total). Genetic variation across locations varied considerably, with a sequence type diversity (h)
283 ranging from 0.273 in Calafuria to 0.890 in Minorca, and a nucleotide diversity (π) ranging from
284 0.003 in San Fruttuoso to 0.071 in Elba (Table 2).

285

286 The two phylogenetic tree reconstruction methods, Maximum Parsimony (MP) and Bayesian

287 Inference (BI), produced similar trees for the 51 ITS-1 sequence types so that only the BI consensus
288 unrooted tree is presented, with both BI posterior probability values and bootstrap values greater
289 than 50% for MP (Figure 2). Four highly supported clades (with BI posterior probability >0.95)
290 could be identified: one consisting of all but one Minorca sequence types (10, 11, 12, 13, 14, 15),
291 one containing sequence types found exclusively in the Tuscany archipelago samples (Giannutri,
292 Elba and Argentario; 30, 31, 32, 45); one containing sequence types found exclusively in Giannutri
293 (34, 35, 36, 37); and one including two sequences found in Sardinia (23) and Medes (8) (Figure 2).

294

295 *Population genetic analysis*

296 Testing the significance of the stepwise clustering procedure performed in STRUCTURE resulted in a
297 separation of the samples into 2 clusters (cluster 1: Medes, Minorca, Plane, Sardinia, San Fruttuoso,
298 Calafuria and Korcula; cluster 2: Argentario, Giannutri, Elba and Palinuro, $\Delta K = 104.5$, Figure 3).
299 The lowest proportion of membership to a particular cluster is 0.563 in cluster 2 for Calafuria
300 sample and the highest proportion is 0.973 in cluster 2 for Giannutri sample (Figure 4).

301

302 High levels of genetic differentiation were found among Mediterranean Sea samples for both
303 microsatellite loci and ITS-1 sequences datasets (Figure 5). Genetic differentiation estimates
304 between microsatellites loci and ITS-1 sequences varied greatly (Figure 5) and were not
305 significantly correlated ($P = 0.87$). For the microsatellite dataset, pairwise F_{ST} estimates based on
306 the ENA method ranged from 0.053 (Minorca vs. Planes) to 0.305 (Giannutri vs. Sardinia; data not
307 shown) and all pairwise comparisons were highly significant ($P < 0.001$). Though these pairwise
308 F_{ST} estimates were significantly smaller than estimated from the original dataset (paired t-test, $P <$
309 0.001), they were significantly correlated (Pearson $r = 0.974$, $P < 0.001$).

310 For the ITS-1 sequence dataset, Φ_{ST} ranged from 0.004 (Calafuria vs. Plane) to 0.789 (Calafuria vs.
311 San Fruttuoso) and two out of the 55 pairwise Φ_{ST} estimates were not statistically significant after
312 sequential Bonferroni correction (Calafuria vs. Plane, and Giannutri vs. Argentario; data not
313 shown). For both datasets (microsatellite and ITS-1), genetic differentiation estimates were not
314 significantly correlated with the logarithm of geographic distances (Figure 5; $P = 0.273$ for
315 microsatellites, $P = 0.136$ for ITS-1)

316

317 The AMOVAs conducted among red coral samples using ITS-1 sequence dataset showed a
318 significant differentiation among samples within all defined groups ($P < 0.001$ for all four analyses,
319 Table 4). A significant variance was observed among groups when grouping samples *a priori* based
320 on the phylogenetic results, representing 33% of the total nuclear variance (i.e. four groups:
321 Minorca vs. Tuscany archipelago vs. Sardinia vs. all others; $V_a = 0.813$, $P = 0.002$, Table 4). Based
322 on the existing gaps in the distribution of the species (i.e. seven groups) or based on the results of
323 the clustering analysis, the nuclear variance attributed among the groups was not significant ($V_a =$
324 0.483 , $P = 0.109$; $V_a = 0.061$, $P = 0.120$, respectively; Table 4). Using the microsatellite dataset, the
325 nuclear variance distributed among groups was not significant except for the grouping based on the
326 clustering analysis ($V_a = 0.065$, $P = 0.020$) and more than 80% of the total variance was observed
327 within samples whatever the grouping (Table 4).

328

329 **Discussion**

330

331 Despite new molecular genetic tools and the plethora of information that is now available for
332 population-level processes describing marine species, our knowledge of evolutionary processes
333 governing Anthozoan coral populations is surprisingly poor, mainly due to the problem in accessing
334 appropriate genetic markers in these species (see van Oppen & Gates 2006 for a review). Indeed,

335 whereas in most animal groups the mitochondrial genome provides an appropriate tool for such
336 investigations, the use of mitochondrial DNA in Anthozoa is limited due to its extremely low
337 evolution rates (Romano & Palumbi 1997, Shearer *et al.* 2002, Hellberg 2006, Calderon *et al.*
338 2006). In particular, in *Corallium rubrum*, no sequence variability was observed in the
339 mitochondrial COI and 16S genes (Costantini *et al.* 2003, Calderon *et al.* 2006). Consequently, for
340 anthozoans, nuclear markers have been mostly used for intraspecific studies (van Oppen & Gates
341 2006), with microsatellites and ITS-1 sequences being nowadays predominant (Rodriguez-Lanetty
342 & Hoegh-Guldberg 2002, Maier *et al.* 2005, Magalon *et al.* 2005, Costantini *et al.* 2007).

344 The main result of our study is that *Corallium rubrum* samples in the Mediterranean Sea are highly
345 genetically heterogeneous. However, the two assessed nuclear markers provide different genetic
346 divergence estimates among samples. Polymorphism levels found in *C. rubrum* samples using
347 microsatellites ($H_{exp} = 0.59 - 0.79$) are comparable with those observed in the gorgonian
348 *Pseudopterogorgia elisabethae* ($H_{exp} = 0.09 - 0.78$, Gutierrez-Rodriguez & Lasker 2004) and in the
349 range of those observed for scleractinian corals (Maier *et al.* 2005, $H_{exp} = 0.73 \pm 0.04$; Magalon *et*
350 *al.* 2005, $H_{exp} = 0.75 \pm 0.01$). However, they are much higher than those obtained with allozyme for
351 the same species ($H_{exp} = 0.073 - 0.104$, Abbiati *et al.* 1993), as expected from the higher mutation
352 rates and polymorphism of microsatellite loci compared to allozyme loci (Estoup & Angers 1998).
353 Heterozygote deficits (strong positive deviations from HWE) were detected for all samples and at
354 almost all microsatellite loci. Heterozygosity deficits have frequently been observed in marine
355 invertebrates (Duran *et al.* 2004a; Addison & Hart 2004), including in many shallow-water
356 Anthozoa (Ayre & Hughes 2000; Ridgway *et al.* 2001; Le Goff-Vitry *et al.* 2004). In *Corallium*
357 *rubrum*, the heterozygote deficit observed may result from technical factors, such as the presence of
358 null alleles, or biological factors, such as inbreeding, Wahlund effect or selection. In our case, as in
359 Costantini *et al.* (2007), non amplifying individuals (null homozygotes) were found in all samples
360 suggesting the occurrence of null alleles. However, observed frequency of null homozygotes were
361 significantly smaller than expected if the heterozygote deficiencies were generated only by null
362 alleles under HWE. In a previous study, Costantini *et al.* (2007) showed that heterozygosity
363 deficiencies found in four proximate (less than 100 meters) samples of red coral collected both
364 within San Fruttuoso and Calafuria were explained by the combined occurrence of null alleles
365 (detected by the presence of non amplifying individuals), inbreeding (more inbred individuals were
366 found than under the hypothesis of random mating) and Wahlund effect (revealed using a
367 maximum-likelihood partitioning method). The heterozygote deficiencies observed in the present
368 analysis further suggest that the latter two biological processes co-occur within all Mediterranean
369 populations of red coral.

371 ITS-1 region sequences investigation may be successful in resolving phylogeographic relationships
372 at large spatial scales, such as in other coral studies (Le Goff-Vitry *et al.* 2004, on *Lophelia pertusa*;
373 Rodriguez-Lanetty & Hoegh-Guldberg 2002, on *Plesiastrea versipora*). However, in Anthozoan,
374 ITS-1 sequence markers may suffer from intra-individual rDNA variability overlapping with
375 within-species rDNA variation (Wei *et al.* 2006, Vollmer & Palumbi 2004, Marquez *et al.* 2003).
376 Indeed, although concerted evolution is expected to homogenize all copies of the rDNA families
377 within individuals (Takabayashi *et al.* 1998), intra-individual variability has been detected in
378 various scleractinian species through cloning and sequencing of ITS PCR products (Vollmer &
379 Palumbi 2004, Ridgway & Gates 2006). For the gorgonian *Corallium rubrum*, we observed 17.5%
380 of unreadable sequences (due to the presence of 1-3 doublet chromatogram peaks or a frame shift in
381 the sequences relative to indels), which may be interpreted as cases of intra-individual
382 polymorphism. In order to avoid including cloning errors and intragenomic variants as sources of

383 additional sequence types (van Oppen & Gates 2006, but see Vollmer & Palumbi 2004 concerning
384 scleractinians corals), we have decided to exclude the individuals showing intra-individual
385 variability from the analyses rather than clone and sequence ITS PCR products. Though excluding
386 individuals showing intra-individual variability (i.e heterozygotes) may reduce the overall
387 frequency of rare alleles, making samples more heterogeneous than they truly are, the fact that the
388 percentage of excluded individuals vary greatly among samples (from 0 to 59%), with the highest
389 values observed in samples where the indels were observed among individuals (Minorca, Sardinia,
390 San Fruttuoso and Giannutri), suggests that the sequences carrying indels were found at very low
391 frequency elsewhere than currently observed, minimizing the bias of our method.

392
393 Despite the intragenomic variation of the ITS-1 and the overestimated genetic differentiation among
394 samples resulting from our method to handle it, some clear patterns are drawn from the
395 phylogenetic analyses. Minorca and Sardinia samples both showed a high genetic variability (0.8 –
396 0.9 sequence type diversity, 1.3 - 3.3% nucleotide diversity) with seven and six private sequence
397 types respectively. Phylogenetic reconstructions showed that Minorca sample appear the most
398 divergent, as expected from its particular insular situation and the complex atmospheric and
399 hydrological systems observed in the Balearic Islands (La Violette *et al.* 1990). Sardinia appears
400 less divergent from the coastal samples compared to Minorca, as expected from its closer location
401 to Corsica and Ligurian coastlines. However, for both islands, we could not propose hypothesis
402 explaining patterns of genetic divergence, neither date the origin of divergence. Indeed, ITS-1
403 region is a nuclear marker experiencing recombination and, to our knowledge, no proper
404 evolutionary rate has been defined in Anthozoa for this marker.

405
406 *Corallium rubrum* analysis revealed a high degree of genetic differentiation between samples
407 throughout the North Western Mediterranean Sea, with apparent genetic homogeneity within the
408 Tuscan archipelago (revealed using ITS-1 sequences, though microsatellite revealed significant
409 differentiation). Microsatellites and ITS-1 sequences showed no correlation between genetic
410 differentiation and geographical distance among the analysed samples. In some cases, for ITS-1
411 sequence data, small geographical distances were associated with relatively small F_{ST} estimates,
412 such as was found when comparing the Argentario and Giannutri samples using ITS-1. In other
413 cases, F_{ST} estimates were large despite geographical proximity, such as was found when comparing
414 the Calafuria and Elba samples for both markers. Such lack of correlation between pairwise F_{ST}
415 estimates and geographical distances may support an island model of dispersal, where larvae move
416 from one area to another with equal probability, rather than an isolation by distance model, where
417 the probability of dispersal between sites declines with increasing geographical distance.

418
419 While microsatellite data showed no correlation between F_{ST} estimates and geographic distances
420 ranging from 30 to 2650 km, Costantini *et al.* (2007) have shown the occurrence of significant
421 genetic structuring at spatial scales of ten meters with a significant isolation by distance at the
422 spatial scale of less than one kilometer, suggesting that larval dispersal in red coral is highly
423 restricted (likely few meters). Therefore, due to their high variability and polymorphism,
424 microsatellites likely appear useless in revealing a clear genetic structuring pattern at the
425 Mediterranean scale. Indeed, microsatellites and ITS-1 are non-coding nuclear markers that should
426 reveal the same patterns under neutrality. However, microsatellites based F_{ST} estimates appeared
427 much smaller as compared to ITS-based estimates (Figure 5). This discrepancy is likely due to allele
428 size homoplasy typical of microsatellites (identity by state but not by descent) resulting from their
429 high mutation rates and allele size range constraints (Goldstein *et al.* 1995, Slatkin 1995). Similar
430 results have been obtained when using loci differing in heterozygosities (O'Reilly *et al.* 2004) or

431 when using highly polymorphic loci over spatial scales similar to the geographical range of the
432 species (Peijnenburg *et al.* 2006).

433
434 Overall, the AMOVAs conducted on the ITS-1 sequences indicated a partitioning of the total
435 genetic variability in four groups: one main group gathering samples belonging to the
436 western/Ligurian Sea (Medes, Plane, San Fruttuoso, Calafuria samples), a second main group of
437 samples from the Tyrrhenian/eastern Seas (Tuscany archipelago, Palinuro and Korcula samples),
438 and two other groups representing an island sample each: Minorca and Sardinia respectively. Using
439 microsatellites data, a subdivision in two clusters identified with a clustering analysis method was
440 confirmed by the AMOVA. However, samples within clusters were not homogeneous (high genetic
441 differentiation for all pairwise comparisons was found), and these results are likely biased by
442 microsatellite size homoplasmy (see previously). Strong patterns of genetic divergence over the North
443 Western Mediterranean Sea have already been reported for other marine invertebrates, and mainly
444 result from large or medium-scale hydrodynamic process or by geomorphologic characteristics of
445 Mediterranean Sea. Duran *et al.* (2004a, 2004b), using both microsatellite loci and ITS sequences,
446 showed that Mediterranean populations of the marine sponge *Crambe crambe* are highly divergent
447 due to the restricted gene flow and follow the isolation by distance model. In this sessile
448 invertebrates, currents and behaviour of the lecithotrophic larvae have a strong influence on the
449 effective larval dispersal, as it may be the case for red coral. Similarly, Lejeune & Chevaldonné
450 (2006) have found high genetic structuring at the Mediterranean scale in two brooding cave-
451 dwelling mysids. While restricted gene flow with isolation by distance explains the patterns
452 observed at the finest scale, habitat disjunction (natural fragmentation) and coastal geomorphology
453 strongly influence population structuring since, in these mysids, dispersal is only possible at the
454 adult stage.

455
456 The results obtained in this study on population genetics of *Corallium rubrum* in the Mediterranean
457 Sea showed the occurrence of a strong large scale structuring and the saturation of the microsatellite
458 loci as compared to the ITS-1 sequences. Lack of clear patterns at the Mediterranean scale together
459 with the results on small scale structuring from Costantini *et al.* (2007) have major implications for
460 the conservation and the sustainable management of this species. Indeed, populations of *C. rubrum*
461 are genetically differentiated at the small scale so that management plans have to be developed at
462 the local scale. Our findings also suggest that, for species with restricted dispersal, absence of clear
463 patterns of genetic structuring over large spatial scales derived from highly polymorphic markers
464 such as microsatellite loci should be interpreted with caution. In fact, chaotic genetic structure at
465 large scales may reveal underlying small scale genetic structuring. Use of different markers (e.g.
466 nuclear or mitochondrial sequences) together with analyses at small spatial scales may help
467 understanding the effective larval dispersal and prevent erroneous interpretation of the genetic
468 patterns observed.

469
470 These results, together with studies on demography (e.g. Santangelo & Abbiati 2001; Garrabou &
471 Harmelin 2002; Torrents *et al.* 2005) and reproductive structure (Santangelo *et al.* 2003) have
472 important consequences for red coral populations resilience. Indeed, an increase of natural and
473 anthropogenic source of mortality such as over harvesting (Santangelo & Abbiati 2001) or water
474 temperature anomalies (Cerrano *et al.* 2000; Garrabou *et al.* 2001) could lead to the disappearance
475 of red coral in shallow water habitats, not only along the Mediterranean continental coasts (Torrents
476 *et al.* 2005) but also along the Mediterranean islands. Further studies that include additional
477 Mediterranean and Atlantic regions, as well as deep sea and peripheral populations of red coral are

478 needed to identify bio-geographical discontinuities and refugia populations of this highly valuable
479 species.
480

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675

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688

689 **Figure Legends:**

690

691 **Figure 1:** Location of the eleven *Corallium rubrum* samples (represented by black dots). Sample
692 names are given based on the name of the proximate island or based on the proximate city. Minorca
693 (MIN) and Medes (MED) are located in the Balearic Sea; Sardinia (SAR) is located in the Sardinian
694 Sea; Plane (PLA), San Fruttuoso (SAN) and Calafuria (CAL) are located in the Ligurian Sea; Elba
695 (ELB), Giannutri (GIA) and Argentario (ARG) are located in the Tuscan archipelago, north
696 Tyrrhenian Sea; Palinuro (PAL) is located in the southern Tyrrhenian Sea; Korcula (KOR) is
697 located in the Adriatic Sea. Dominating surface currents are indicated as grey arrows (based on
698 Pinardi & Masetti 2000).

699

700 **Figure 2:** Unrooted phylogenetic tree of 51 ITS-1 sequence types of *Corallium rubrum* using
701 Bayesian inference (BI). The MP tree showed the same pattern. Posterior probability (BI) and
702 bootstrap values are indicated (BI/MP). Numbers in brackets denote the number of individuals per
703 sample for each corresponding clade.

704

705 **Figure 3:** Values of ΔK , calculated as in Evanno *et al.* (2005) and based on the log likelihood of the
706 data given by STRUCTURE version 2.2 for each number of cluster assumed (K).
707

708 **Figure 4:** Results of the clustering analysis conducted in STRUCTURE 2.2. In the bar plot, each of
709 the 370 individuals is represented by a vertical bar indicating its estimated proportion of
710 membership to each cluster (represented by different colours). Under the bar plot is indicated the
711 mean proportion of membership of each sample to each cluster.
712

713 **Figure 5:** Relationship between genetic differentiation estimates and the logarithm of geographical
714 distance among *Corallium rubrum* samples for both microsatellite and ITS-1 markers.
715

716 **Authors Information Box**

717 This paper was part of the PhD work of Dr Federica Costantini supervised by Prof. Marco Abbiati,
718 which aimed to focus on population genetics and molecular systematic of marine invertebrates in
719 coastal habitats, mainly for biodiversity conservation purposes. Dr Cécile Fauvelot is a molecular
720 ecologist who has worked on both coral reef fish and rainforest butterfly population genetics and
721 contributed to this work during her post doctoral position in Ravenna.
722
723

724 **Table 1:** Summary of genetic diversity at five microsatellite loci from *Corallium rubrum* samples;
725 n: number of sampled individuals; N: number of genotypes per loci; A: allelic richness based on 16
726 ind.; H_{obs} : observed heterozygosity; H_{exp} : expected heterozygosity; F_{IS} : Weir and Cockerham's
727 (1984) estimate of Wright's (1951) fixation index (bold type indicate significant deviations from
728 HWE after standard Bonferroni correction); R : null allele frequency based on Dempster *et al.*
729 (1977) estimator.

	MED	MIN	PLA	SAR	SAN	CAL	ARG	GIA	ELB	PAL	KOR
<i>n</i>	24	24	20	28	50	50	48	36	32	24	34
COR9											
N	23	21	18	26	46	47	44	35	30	23	32
A	12.3	9.1	11.8	9.3	7.5	12.2	7.6	7.8	7.1	6.7	5
H_{obs}	0.39	0.24	0.22	0.31	0.26	0.21	0.2	0.34	0.37	0.22	0.12
H_{exp}	0.86	0.8	0.87	0.84	0.62	0.89	0.79	0.85	0.74	0.73	0.72
F_{IS}	0.56	0.71	0.76	0.64	0.58	0.77	0.75	0.60	0.52	0.71	0.83
R	0.29	0.42	0.42	0.35	0.32	0.40	0.40	0.30	0.29	0.34	0.40
COR15											
N	24	24	18	28	47	50	48	35	32	24	34
A	3.6	4.8	1	3	3	3.6	3	2.4	3.2	3.7	3.8
H_{obs}	0.5	0.54	0	0.46	0.36	0.68	0.42	0.37	0.25	0.83	0.41
H_{exp}	0.53	0.5	0	0.44	0.53	0.67	0.56	0.32	0.34	0.67	0.62
F_{IS}	0.08	-0.06	-	-0.04	0.33	-0.001	0.27	-0.13	0.28	-0.22	0.35
R	0.01	0.00	0.32	0.02	0.22	0.00	0.08	0.09	0.09	0.00	0.11
COR46											
N	22	22	18	16	20	42	46	35	29	24	29
A	9.6	12.4	6	9	7.9	7.7	8.5	3.9	4.6	9.5	8
H_{obs}	0.27	0.5	0.17	0	0.1	0.48	0.37	0.08	0.24	0.79	0.31
H_{exp}	0.87	0.87	0.75	0.86	0.82	0.74	0.81	0.48	0.55	0.83	0.83
F_{IS}	0.70	0.44	0.79	1	0.88	0.94	0.55	0.83	0.57	0.07	0.64
R	0.39	0.28	0.42	0.71	0.78	0.52	0.29	0.32	0.33	0.03	0.41
COR48											
N	21	23	18	24	47	48	48	36	29	24	34
A	11.1	15.3	8.8	8.8	5.8	6.6	9.8	5.4	8.2	10.4	8.2
H_{obs}	0.43	0.96	0.33	0.17	0.25	0.27	0.42	0.64	0.27	0.92	0.56
H_{exp}	0.85	0.91	0.79	0.82	0.66	0.73	0.82	0.65	0.8	0.81	0.78
F_{IS}	0.51	-0.03	0.60	0.80	0.62	0.64	0.50	0.04	0.67	-0.11	0.30
R	0.34	0.05	0.35	0.47	0.32	0.31	0.23	0.03	0.38	0.00	0.12
COR58											
N	23	22	18	27	45	46	46	34	29	21	32
A	9	8.4	5	2.7	6.6	13.3	7.5	6.4	7.8	10.4	7.1
H_{obs}	0.39	0.41	0.28	0	0.2	0.5	0.54	0.29	0.65	0.67	0.34
H_{exp}	0.82	0.8	0.76	0.14	0.76	0.89	0.59	0.63	0.81	0.88	0.67
F_{IS}	0.54	0.51	0.65	1	0.74	0.45	0.09	0.54	0.20	0.26	0.50
R	0.28	0.30	0.37	0.26	0.41	0.28	0.11	0.29	0.21	0.25	0.27
Multilocus											
A	9.1	10	6.5	6.5	6.2	8.7	7.3	5.2	6.2	8.1	6.4
H_{obs}	0.4	0.53	0.2	0.19	0.23	0.34	0.39	0.35	0.36	0.68	0.35
H_{exp}	0.79	0.78	0.63	0.62	0.68	0.78	0.71	0.59	0.65	0.78	0.72
F_{IS}	0.51	0.34	0.70	0.71	0.66	0.57	0.46	0.42	0.46	0.15	0.53

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733 **Table 2:** Distribution of sequence types and genetic diversity in *Corallium rubrum* samples;
 734 number of individuals amplified per sample (N), number of individuals per sample with analysed
 735 sequence (n), total number of sequence type (H), sequence type diversity (*h*, Nei 1987), nucleotide
 736 diversity (π , Nei 1987).
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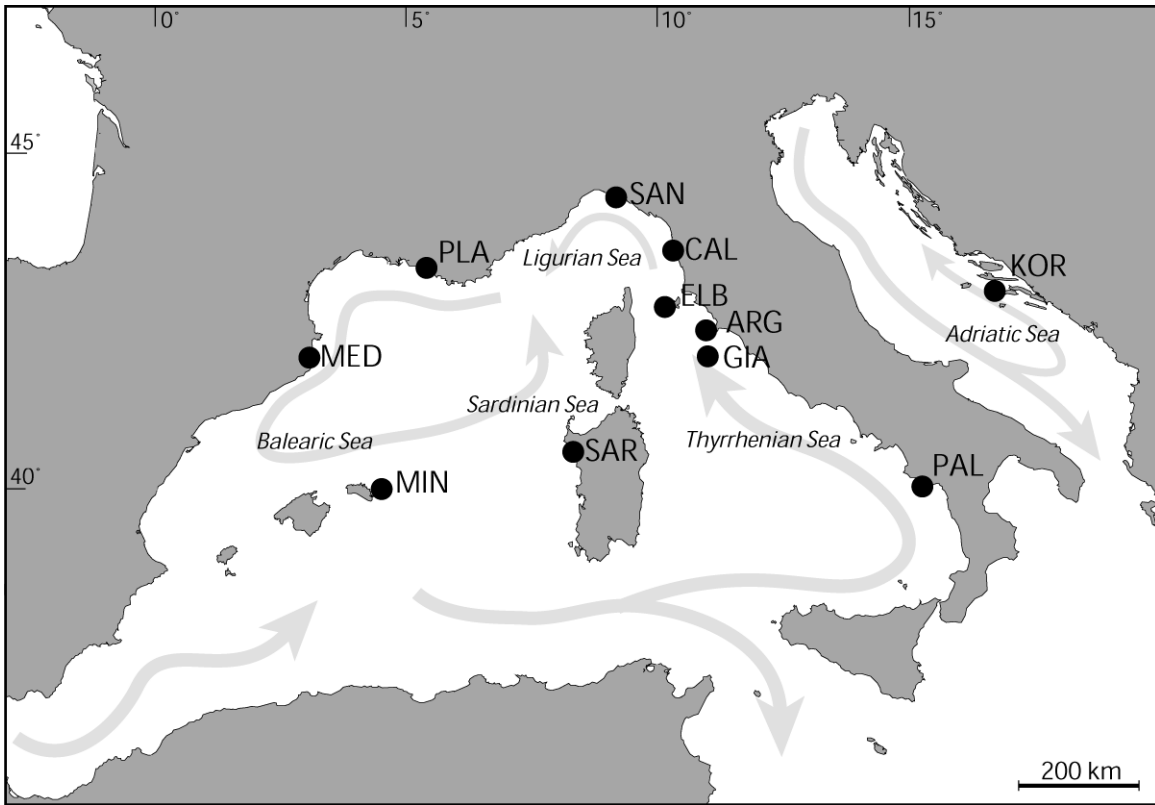
	MED	MIN	PLA	SAR	SAN	CAL	ARG	GIA	ELB	PAL	KOR	Total
N	24	15	20	17	22	34	34	36	20	24	34	280
1	11		1									12
2	1											1
3	1											1
4	1											1
5	4					4	8	1	4	5	2	28
6	3		1							4		8
7	1											1
8	1											1
9		1										1
10		1										1
11		3										3
12		1										1
13		1										1
14		3										3
15		1										1
16			2									2
17			15			28						43
18			1									1
19				1								1
20				1								1
21				5								5
22				2								2
23				1								1
24				1								1
25					7							7
26					2							2
27						1						1
28							2					2
29							15	7				22
30							2					2
31							1					1
32							4	4	7			15
33							2					2
34								1				1
35								2				2
36								1				1
37								1				1
38								1				1
39								1				1
40								2				2
41									1			1
42									1		26	27
43									2			2
44									1			1
45									1			1
46										1		1
47										4		4
48										1		1
49										4	2	6
50										1	1	2
51											1	1
n	23	11	20	11	9	33	34	21	17	20	32	231
H	8	7	5	6	2	3	7	10	7	7	5	51
<i>h</i>	0.747	0.89	0.442	0.8	0.39	0.273	0.747	0.862	0.794	0.853	0.341	0.92
π	0.01	0.033	0.005	0.013	0.003	0.005	0.046	0.044	0.071	0.012	0.006	0.046

804 **Table 4:** Analysis of molecular variance (AMOVA) among samples of *Corallium rubrum* using
805 ITS-1 dataset and microsatellite dataset. Red coral samples were grouped according to A) their sea
806 of origin (six groups: Balearic Sea, Sardinian Sea, Ligurian Sea, North and Southern Tyrrhenian
807 Sea, Adriatic Sea), B) the existing gaps in the distribution of the species (seven groups: Minorca;
808 Sardinia; Medes; Plane and San Fruttuoso; Calafuria, Argentario, Giannutri and Elba; Palinuro;
809 Korcula), C) the phylogenetic results (four groups: Minorca; Sardinia; Tuscan archipelago samples
810 and all the other samples) and D) the clustering analysis results (two groups: Minorca, Sardinia,
811 Medes, Plane, San Fruttuoso, Calafuria and Korcula; Argentario, Giannutri, Palinuro and Elba). * P
812 < 0.05 , *** $P < 0.001$.
813

Source of variation	ITS - 1			Microsatellites		
	d.f.	Variance components	%	d.f.	Variance components	%
(A) Six basins						
Among basins	5	1.187	8.66	5	0.059	3.12
Among samples within basins	5	0.872	40.42***	5	0.300	15.95***
Within samples	220	1.098	50.93***	729	1.521	80.93***
(B) seven groups						
Among groups	6	0.483	21.77	6	0.049	2.62
Among samples within groups	4	0.637	28.71***	4	0.309	16.45***
Within samples	220	1.098	49.52***	729	1.521	80.93***
(C) Four groups						
Among groups	3	0.813	33.19***	3	0.06	3.27
Among samples within groups	7	0.538	21.96***	7	0.31	16.31***
Within samples	220	1.098	44.85***	729	1.52	80.41***
(D) Two groups						
Among groups	1	0.061	2.82	1	0.065	3.42*
Among samples within groups	9	1.002	46.36***	9	0.315	16.59***
Within samples	220	1.098	50.82***	729	1.521	79.99***

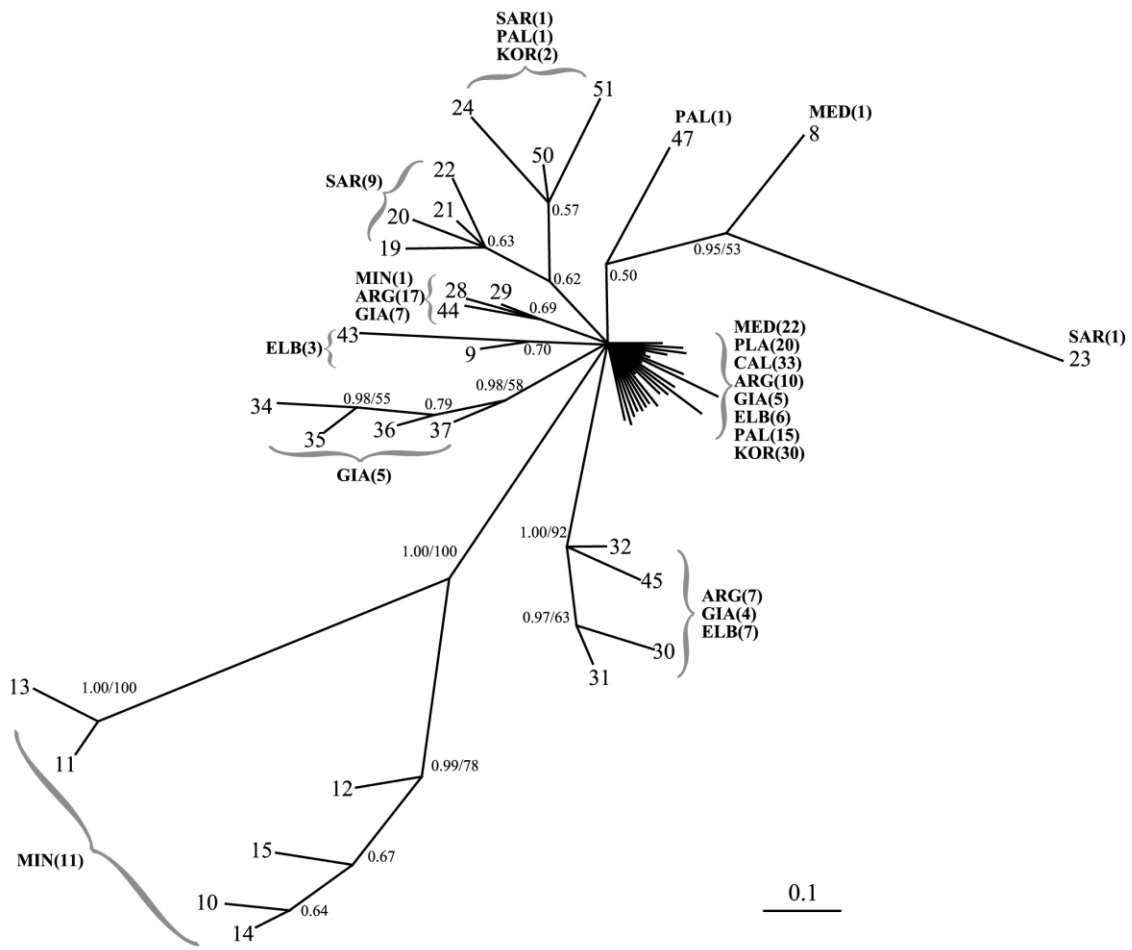
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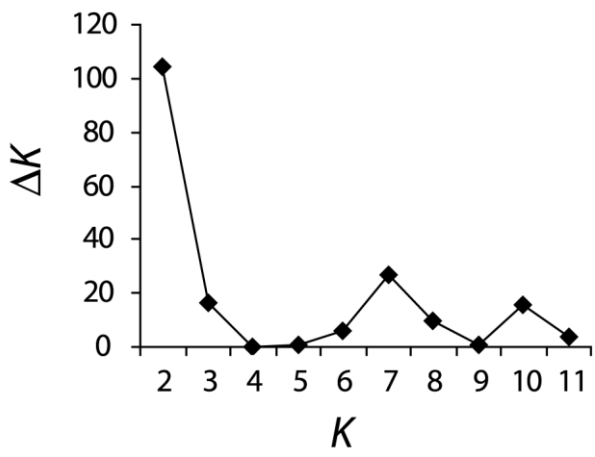
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Figure 1



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Figure 2



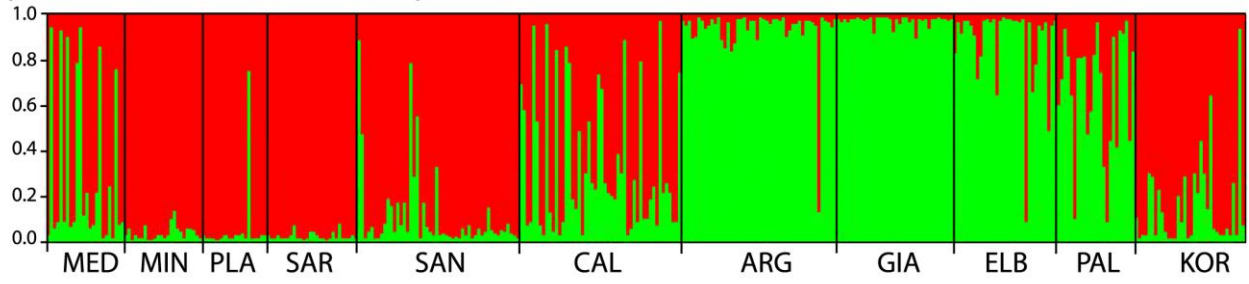
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831 **Figure 3**

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Proportion of individual membership



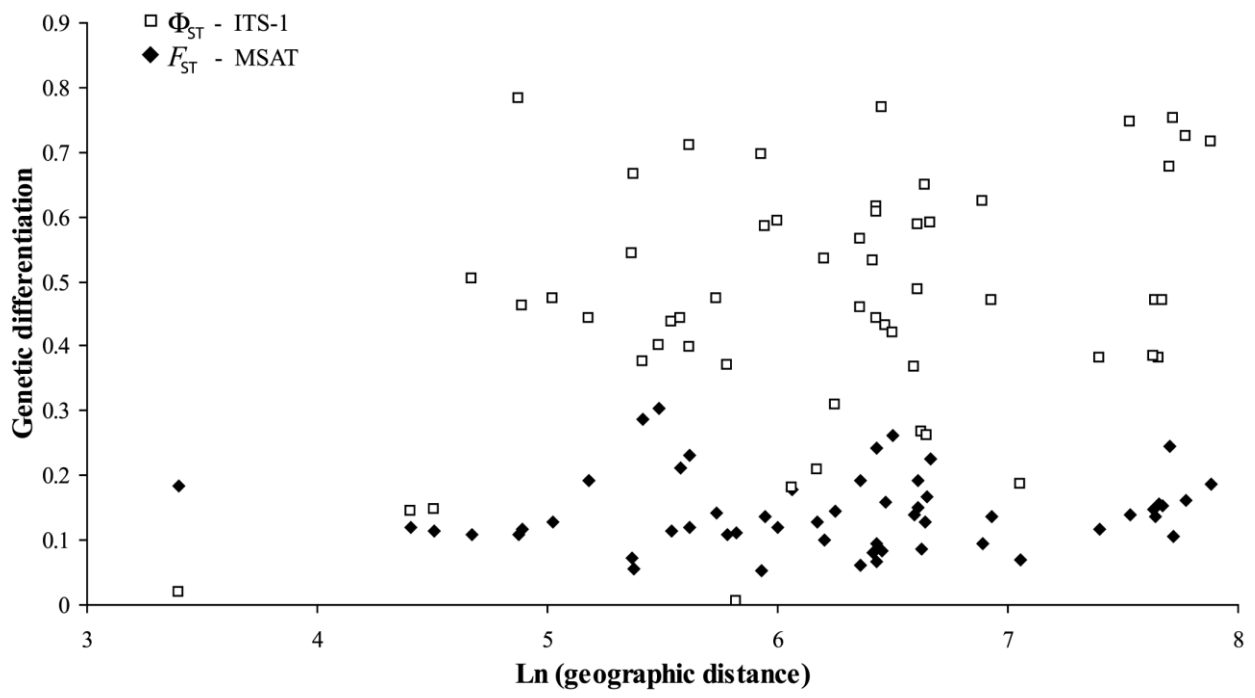
Mean proportion of sample membership

833	Cluster 1	0.667	0.958	0.940	0.964	0.843	0.563	0.064	0.027	0.131	0.418	0.841
	Cluster 2	0.333	0.042	0.060	0.036	0.157	0.437	0.936	0.973	0.869	0.582	0.159

834

835 **Figure 4**

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838 **Figure 5**

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